Nuclear localisation of 53BP1 is regulated by phosphorylation of the nuclear localisation signal

Running title: Identification of 53BP1's nuclear localisation signal.

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Abstract

Background information: Repair of damaged DNA is essential for maintaining genomic stability. TP53-binding protein 1 (53BP1) plays an important role in repair of the double strand DNA breaks. Nuclear localisation of 53BP1 depends on importin β and nucleoporin 153 but the type and location of 53BP1 nuclear localisation signal has yet to be determined.

Results: We show here that nuclear import of 53BP1 depends on two basic regions, namely 1667-KRK-1669 and 1681-KRGRK-1685, that both are needed for importin binding. Lysine 1667 is essential for interaction with importin and its substitution to arginine reduced nuclear localisation of 53BP1. Further, we have found that, CDK1-dependent phosphorylation of 53BP1 at S1678 impairs importin binding during mitosis. Phosphorylation mimicking mutant S1678D showed reduced nuclear localization suggesting that phosphorylation of the NLS interferes with nuclear import of the 53BP1

Conclusions: We show that 53BP1 contains a classical bipartite nuclear localisation signal, 1666-GKRKLITSEEERSPAKRGRKS-1686, which enables the importin-mediated nuclear transport of 53BP1. Additionally, we find that posttranslational modification within the NLS region can regulate 53BP1 nuclear import.

Significance: Our results indicate that nuclear import of 53BP1 is regulated by posttranslational modifications. Mutations in this region identified in cancer cells can impair nuclear localisation of 53BP1 and thus contribute to development of genomic instability.

Introduction

The DNA damage response (DDR) is a network of pathways protecting against genomic instability by detecting DNA damage and taking the appropriate actions including activation of the cell cycle checkpoint and DNA repair. The repair of double strand DNA breaks (DSBs) occurs mainly through non-homologous end joining (NHEJ) or homologous recombination (HR), the later uses a template to enable error free repair. The need for a template restricts HR to the S and G2 phase of the cell cycle when the sister chromatid is present (Jackson and Bartek 2009). Choosing the appropriate repair pathway is essential for maintaining genomic stability. Controlling resection at the site of DSBs is important for pathway choice, resection stimulates HR while at the same time inhibits NHEJ. 53BP1 and its binding partners RIF1 and PTIP inhibit resection of DNA ends, while Breast cancer type 1 susceptibility protein (BRCA1) competes with 53BP1 and promotes resection (Bunting, et al. 2010; Callen, et al. 2013; Zimmermann, et al. 2013).

53BP1 acts as a molecular platform for the recruitment of DNA damage response proteins to the sites of DSBs. The N-terminus of 53BP1 contains 28 S/TQ sites that can be phosphorylated by ATM and/or ATR after the occurrence of DNA damage, enabling the interaction with RIF1 and PTIP (Zimmermann and de Lange 2014). The central part (residues 1220-1711) comprising of the oligomerization domain, Tudor domain and the ubiquitination-dependent recruitment (UDR) motif represent a minimal focus-forming region that is sufficient for localization of 53BP1 to the chromatin flanking the DSB. The Tudor domain binds dimethylated histone H4K20 while the UDR motif binds histone H2A ubiquitinated by RNF8 on K13/K15 (Botuyan, et al. 2006; Fradet-Turcotte, et al. 2013) (Figure1A). The BRCT domain localized at the C-terminus binds to yH2AX and is not essential for, but further improves the efficiency of 53BP1

recruitment to DNA damage sites and promotes DNA repair mainly in heterochromatin regions (Baldock, et al. 2015; Kleiner, et al. 2015). During mitosis 53BP1 recruitment to DNA damaged site is inhibited by PLK1 and CDK1 mediated phosphorylation of the UDR motif to prevent the fusion of telomeres (Benada, et al. 2015; Giunta, et al. 2010; Orthwein, et al. 2014).

After translation in the cytoplasm, 53BP1 is imported into the nucleus to execute its important functions in DNA repair. Nuclear transport depends on nuclear pore complexes that consist out of nucleoporins. The nuclear pore complex has a cytoplasmic region, a trans-membrane region and a nuclear region that appear as ring like structures. On the nuclear side are eight extended filaments that join together and form the nuclear basket. Similarly filaments extent into the pore creating a selective environment through which proteins have to pass (Knockenhauer and Schwartz 2016). Small molecules are able to diffuse in and out of the nucleus, while bigger molecules, including 53BP1, require active transport. For active transport the protein needs to be recognized by nuclear transport receptors and transporters, which use a RANGTP gradient providing the energy for directed transport of their cargo (Cavazza and Vernos 2015). The direction of the transport can occur both into and out of the nucleus depending on where the cargo binds the nuclear receptor. Proteins containing a NLS bind nuclear transporters in the cytoplasm and are released in the nucleus upon binding to RANGTP while proteins with a nuclear export signal (NES) bind in the nucleus and are released in the cytoplasm when RANGTP is hydrolysed. Nuclear import of 53BP1 depends on the nuclear transporter importin β and nucleoporin Nup153 kDa (NUP153) (Lemaitre, et al. 2012; Moudry, et al. 2012). Depletion of NUP153 negatively affects NHEJ and reduces both the nuclear localisation and sumoylation of 53BP1 (Duheron, et al. 2017). NUP153 forms part of the nuclear basket of nuclear core complexes and two distinct nuclear import pathways converge on it, transportin and importin mediated transport (Shah and Forbes 1998). Importin β can directly recognize it substrates but more often the recognition occurs through adaptor proteins (Cingolani, et al. 1999; Lee, et al. 2003). The most common importin β adaptor is importin α, which binds importin β through its IBB domain and proteins containing a NLS. Importin α binds a classical mono or bipartite NLS consisting out of 1 or 2 clusters of basic residues, respectively (Conti, et al. 1998). Post-translational modifications within or adjacent to a NLS can affect importin α binding and thereby the nuclear import. Phosphorylations have been reported to both increase and decrease the binding to importin α while acetylation generally inhibits the binding with importin α (di Bari, et al. 2006; Harreman, et al. 2004; Jeong, et al. 2015; Madison, et al. 2002; Nardozzi, et al. 2010).

It is well established that the minimal focus forming region of 53BP1 enters the nucleus, however the exact location the NLS has not been mapped. Here we show that 53BP1 contains a classical bipartite NLS, 1666-G**KRK**LITSEEERSPA**KRGRK**S-1686, which enables the interaction with importin α and importin β. In addition, we explore the impact of post-translational modifications on 53BP1 nuclear import.

Results

53BP1 contains a classical bipartite NLS.

Previous reports showed that the nuclear import of 53BP1 occurs through importin β. As binding of importin β to a classical NLS is commonly mediated by importin α we tested the interaction of 53BP1 with importin α. Immunoprecipitation of GFP-53BP1 revealed its interaction with several isoforms of importin α implicating the presence of a classical NLS (Figure 1B). Using cNLS mapper and Eukaryotic Linear Motif software, we identified several sequences in 53BP1 that could function as classical NLS including two potential monopartite NLSs and one bipartite NLS that overlaps with one of the mono partite NLSs (Table 1). Deletion of all of these predicted NLSs in GFP 53BP1 Δ1626-1692 (ΔNLS1-3) results in the cytoplasmic retention of 53BP1 (Figure 1C). The putative bipartite NLS in 53BP1, 1666-G**KRK**LITSEEERSPA**KRGRK**S-1686, received the highest score in cNLS mapper. We have found that alanine substitution of either one of the basic amino acid stretches within this bipartite NLS (ΔNLS2 and ΔNLS3) prevents nuclear import of 53BP1 while the alanine substitution of the predicted monopartite NLS, 1624-EGKRKRRSNVS-1634, does not influence 53BP1 localisation (ΔNLS1) (Figure 1C). In line with these findings, we detected interaction between GFP-53BP1-WT and importin β, while alanine substitution of the basic amino acids in NLS2 and NLS3 regions resulted in the loss of this interaction (Figure 1D).

A bipartite NLS is sufficient for nuclear import of 53BP1

To test if the bipartite NLS present in 53BP1 is sufficient to mediate nuclear localisation, we expressed a GFP fused to the bipartite NLS in U2OS cells. We found that GFP-NLS localizes exclusively to the nucleus whereas GFP-FLAG showed both cytoplasmic and nuclear distribution (Figure 2A). In addition, GFP-NLS strongly co-immunoprecipitated importin β while GFP-FLAG did not interact with importin β (Figure 2B). Together these findings indicate that 53BP1 contains a classical bipartite NLS 1666-G**KRK**LITSEEERSPA**KRGRK**SA-1686 that is sufficient for importin binding and 53BP1 nuclear localisation.

K1667 integrity is required for importin binding and nuclear localisation of 53BP1

After mapping the NLS of the 53BP1 we screened available proteomic databases to search for posttranslational modifications that could potentially regulate the nuclear import of 53BP1. Since acetylation of lysine residues within a NLS can directly disrupt the interaction with importin α and thereby inhibit nuclear transport (Madison, et al. 2002) we set out to functionally test the acetylation of K1667 that was previously reported by proteomic screens (Hornbeck, et al. 2015). To this end, we mutated K1667 to arginine and glutamine, mimicking the non-acetylated and the acetylated lysine, respectively. Consistent with the possibility that acetylation could interfere with importin binding to the NLS, we noted that the acetylation-mimicking K1667Q mutant did not bind importin β. However, K1667R mutant also did not bind importin β (Figure 3A). Likewise, 53BP1-K1667R mutant showed strongly impaired nuclear localisation indicating that presence of the first lysine is essential for the nuclear localisation of 53BP1 (Figure 3B and 3C). To test the effect of acetylation on importin binding

we performed *in vitro* acetylation of isolated GFP NLS and subsequently pulled down proteins from the cell extract (Figure 3D). Although we detected strong acetylation of the GFP NLS (but not of the GFP alone) we did not observe differences between importin binding to the non-acetylated NLS and acetylated NLS. We conclude that the integrity of the NLS is essential for interaction with importin but its acetylation does not significantly affect the importin binding.

Regulation of 53BP1 nuclear import by phosphorylation of the NLS

Phosphorylation is a common mechanism by which nuclear import is influenced (Nardozzi, et al. 2010). Phosphoproteomic screens have identified multiple phosphorylations of 53BP1, including several modifications that occur specifically during mitosis (Dephoure, et al. 2008; Olsen, et al. 2010) (Figure 4A). In agreement with these studies, we noted that the 53BP1 minimal focus-forming region is phosphorylated in mitotic cells (Figure 4B). We and others have recently reported S1618 as substrate for PLK1 and S1609 and S1678 as major CDK1-dependent phosphorylation sites in the C-terminal part of 53BP1 (Benada, et al. 2015; Orthwein, et al. 2014). In addition, Aurora-B has been shown to phosphorylate S1342 of 53BP1 and to regulate its attachment to kinetochores (Wang, et al. 2017). Here we aimed to test the contribution of phosphorylation events occurring in the NLS to the nuclear transport of 53BP1. To this end, we created an alanine and an aspartate substitution of S1678 mimicking the non-phosphorylated and phosphorylated state of the protein, respectively. Whereas alanine was relatively well tolerated at this position, S1678D mutant showed a clear enrichment of 53BP1 in cytoplasm resulting in a significant decrease of the nuclear/cytoplasmic ratio (Figure 4C and 4D). In addition, 53BP1-S1678D mutant showed dramatically reduced interaction with importin β (Figure 4E). To test if the nuclear import of 53BP1 could be regulated by the cell cycle, we immunoprecipitated endogenous 53BP1 from cells synchronized in G1 or in mitosis and found decreased interaction of importin α with 53BP1 in mitotic cells which is consistent with masking the NLS by phosphorylation during mitosis (Figure 4F). In addition, mitotic exit forced by addition of CDK1 inhibitor resulted in enhanced binding of importin α to 53BP1 compared to the binding during mitosis (Figure 4G). We conclude that interaction between importin β and 53BP1 is inhibited during mitosis by CDK1-dependent phosphorylation of the NLS and is rapidly reconstituted upon mitotic exit.

Discussion

Here we have mapped the nuclear localisation signal of the 53BP1, to the region between amino acids 1666-1686, and explored potential regulatory mechanisms of 53BP1 nuclear import. As the acetylation mimicking and non-acetylatable arginine mutants of K1667 both severely affected interaction with importin β, we conclude that the integrity of the NLS rather than the acetylation status of K1667 is important for the interaction of 53BP1 with importin.

Protein 53BP1 is extensively post-translationally modified at multiple residues including about 60 identified phosphorylation sites. Some of these phosphorylations are induced by DNA damage whereas others may be regulated during the cell cycle (Dephoure, et al. 2008;

Hornbeck, et al. 2015; Olsen, et al. 2010; Stokes, et al. 2007) .The latter group of 53BP1 phosphorylations includes S1618, S1609 and S1678 that are mediated by PLK1 and CDK1, respectively, and occur during mitosis (Benada, et al. 2015; Orthwein, et al. 2014). Since the S1678 localizes to the NLS region we tested the impact of its phosphorylation on 53BP1 localization. Phoshomimicking mutation S1678D inhibits the interaction between 53BP1 and importin α and strongly decreased the nuclear/cytoplasmic ratio of the 53BP1 distribution. During mitosis, 53BP1 recruitment to sites of DNA damage is prevented by a combined action of reduced histone ubiquitination and phosphorylation of its UDR domain (Benada, et al. 2015; Lee, et al. 2014; Orthwein, et al. 2014). This is important for genetic stability and restoring the recruitment of 53BP1 to chromatin during mitosis results in the formation of telomere fusions (Orthwein, et al. 2014). Nuclear OPT bodies containing 53BP1 represent under-replicated regions of genomic DNA and they are formed during mitotic exit concomitantly with formation of the nuclear envelope (Harrigan, et al. 2011; Lukas, et al. 2011). 53BP1 is thought to protect these regions from error-prone repair in G1 until they are eventually repaired during S/G2 phase of the cell cycle (Harrigan, et al. 2011). During mitotic exit, 53BP1 phosphorylation within the UDR domain is removed by PP4C/R3β phosphatase to again allow recruitment of 53BP1 to the sites of DNA damage (Lee, et al. 2014). Similarly, we propose that phosphorylation of S1678 needs to be removed during mitotic exit to allow efficient nuclear import of 53BP1 at time when nuclear envelope is reconstituted. Activity of protein phosphatases that oppose the CDK1-mediated phosphorylations is very high at mitotic exit and results in rapid dephosphorylation of many cellular proteins (Mochida, et al. 2009). One of the candidate phosphatases that could counteract the 53BP1 phosphorylation at S1678 is PP4 that has recently been demonstrated to interact with 53BP1 during completion of cell division (Lee, et al. 2014). Development of a phospho-specific antibody against S1678 of 53BP1 will be needed for testing this possibility at cellular level. In conclusion, we show that 53BP1 contains a classical bipartite nuclear localisation signal, 1666-G**KRK**LITSEEERSPA**KRGRK**S-1686, which enables importin-mediated transport of 53BP1 and is solely sufficient for 53BP1 nuclear import. In addition, we find that posttranslational modification within the NLS region have the potential of regulating 53BP1 nuclear import. Finally, by searching the COSMIC database we noted that somatic mutations occasionally occur within the NLS region of 53BP1 in cancer cells and we speculate that preventing the nuclear localisation of 53BP1 could contribute to development of genomic instability in tumor cells.

Material and Methods

Antibodies. The following antibodies were used in this study: rabbit polyclonal and mouse monoclonal against 53BP1 (sc22760 and sc515841), importin β (sc137016), cyclin A (sc751) from Santa Cruz Biotechnology; histone H3-pS10 (Milipore); Importin α (I1784, Sigma-Aldrich); T7-Tag (clone D9E1X), Acetylated lysine (#9441), phospho-Threonine-Proline (#9391) from Cell Signaling; mouse monoclonal anti-GFP (11814460001, Roche); and Cdc27 (610454, BD Bioscience).

Plasmids. The pcdna4-EGFP-FLAG-53BP1 construct (Benada, et al. 2015) was subjected to site directed mutagenesis to generate pcdna4-EGFP-FLAG-53BP1-ΔNLS1 carrying aa1626-1630 substitution to alanines, pcdna4-EGFP-FLAG-53BP1-ΔNLS2 carrying aa1667-1669 substitution to alanines, pcdna4-EGFP-FLAG-53BP1-ΔNLS3 carrying aa1681-1685 substitution to alanines, pcdna4-EGFP-FLAG-53BP1-ΔNLS1-3 carrying deletion of aa1626-1692 region, pcdna4-EGFP-FLAG-53BP1-S1678A and pcdna4-EGFP-FLAG-53BP1-S1678D. Plasmid coding for GFP-NLS was generated by in frame ligating DNA fragment carrying sequence for 1658-RASMGVLSGKRKLITSEEERSPAKRGRKSA-1687 and stop codon into pcdna4-EGFP. Plasmids pCMVTNT-T7-KPNA1-7 for expression of individual importin alpha variants were a gift from Bryce Paschal (Addgene).

Cell culture. Human osteosarcoma cell line (U2OS) and Human embryonic kidney cells (HEK293) were cultivated in DMEM at 37°C in a humidified atmosphere with 5% CO2. Cells were transfected by 6:1 mixture of linear Polyethylenimine PEI MAX 40 kDa (Polysciences Inc) and plasmid DNA and transfection media was replaced with fresh media after 4 h (U2OS) or overnight incubation (HEK293). For cell cycle synchronization, cells were transfected with plasmid DNA and grown for 24 h prior to cell cycle synchronization by thymidine block. Cells were incubated in thymidine (2 mM) for 20 h and then released into fresh media. After 2 hours, DMSO, RO-3306 (9 μM) or nocodazole (330 nM; Sigma-Aldrich) were added and cells synchronized G1, G2 or mitosis were collected after 18 h. To force mitotic exit, cells were collected by mitotic shake-off and incubated for 1 h with RO-3306.

Immunoprecipitation. Transiently transfected cells were lysed in 500 µl of ice cold IP buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 2.5 mM EGTA, 10 % (v/v) glycerol) supplemented with cOmplete EDTA-free protease inhibitor, PhosSTOP phosphatase inhibitor (Sigma-Aldrich) and EtBr (50 µg/ml)) and sonicated 3×10 s. Cell extracts were cleared by centrifugation at 15.000 rpm for 15 min and incubated at 4°C for 1-2 hours with 25 µl GFP-Trap (ChromoTek). Beads were washed three times with IP buffer, bound proteins were eluted by SDS sample buffer and analyzed by western blotting.

In vitro acetylation and pull down. GFP or GFP-NLS were immunoprecipitated from transfected cells using GFP-Trap, beads were washed once with IP buffer containing 1 M NaCl and once in acetylation buffer (100 mM Tris pH 7.5, 10 % glycerol, 0.1 mM EDTA, 1 mM DTT, 1 mM PMS, 20 µM acetyl-coA). Beads were incubated with 1 µg of P300 HAT domain (Sigma) in acetylation buffer for 1 h at 30°C. Subsequently, beads were incubated with pre cleared cell lysates in IP buffer for 30 min at 4°C. Proteins were eluted by SDS sample buffer, boiled and analyzed by western blotting.

Microscopy. U2OS Cells were grown on glass coverslips, fixed with 4% (w/v) paraformaldehyde in PBS for 10 min, permeabilized with 0.5 % (v/v) TritonX-100 in PBS for 5 min and blocked with 3 % BSA in PBST for 30 min. Next, cells were incubated for 1 h with primary antibodies diluted in PBST, washed with PBST and incubated with secondary antibody for 1 h and washed with PBST. Coverslips were mounted with Vectashield reagent and DAPI (Vector Laboratories). Images were acquired on Leica DM6000 fluorescence microscope with

HCX PL Apo63x/1.40 oil PH3 CS objective (Leica). Quantification of nuclear and cytoplasmic GFP signal was done in ImageJ.

In silico NLS prediction. cNLS mapper and Eukaryotic Linear Motif (ELM) prediction software were used to predict potential nuclear localisation signals. cNLS mapper activity cut off score was set at 3 and a longer linker of bipartite NLS (13-20 amino acids) was only allowed within the terminal 60 amino acids (Dinkel, et al. 2012; Kosugi, et al. 2009).

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Author contributions

PvM and TL carried out experimental work and were involved in data analysis. PvM, ZH and LM designed the experiments and wrote the article.

List of abbreviations

53BP1	p53 binding protein 1
BRCA1	breast cancer type 1 susceptibility protein
Co-IP	CO-Immunoprecipitation
DSB	double strand break
HR	homologous recombination
NHEJ	non-homologous end joining
NES	nuclear export signal
NLS	nuclear localisation signal
UDR	ubiquitination-dependent recruitment

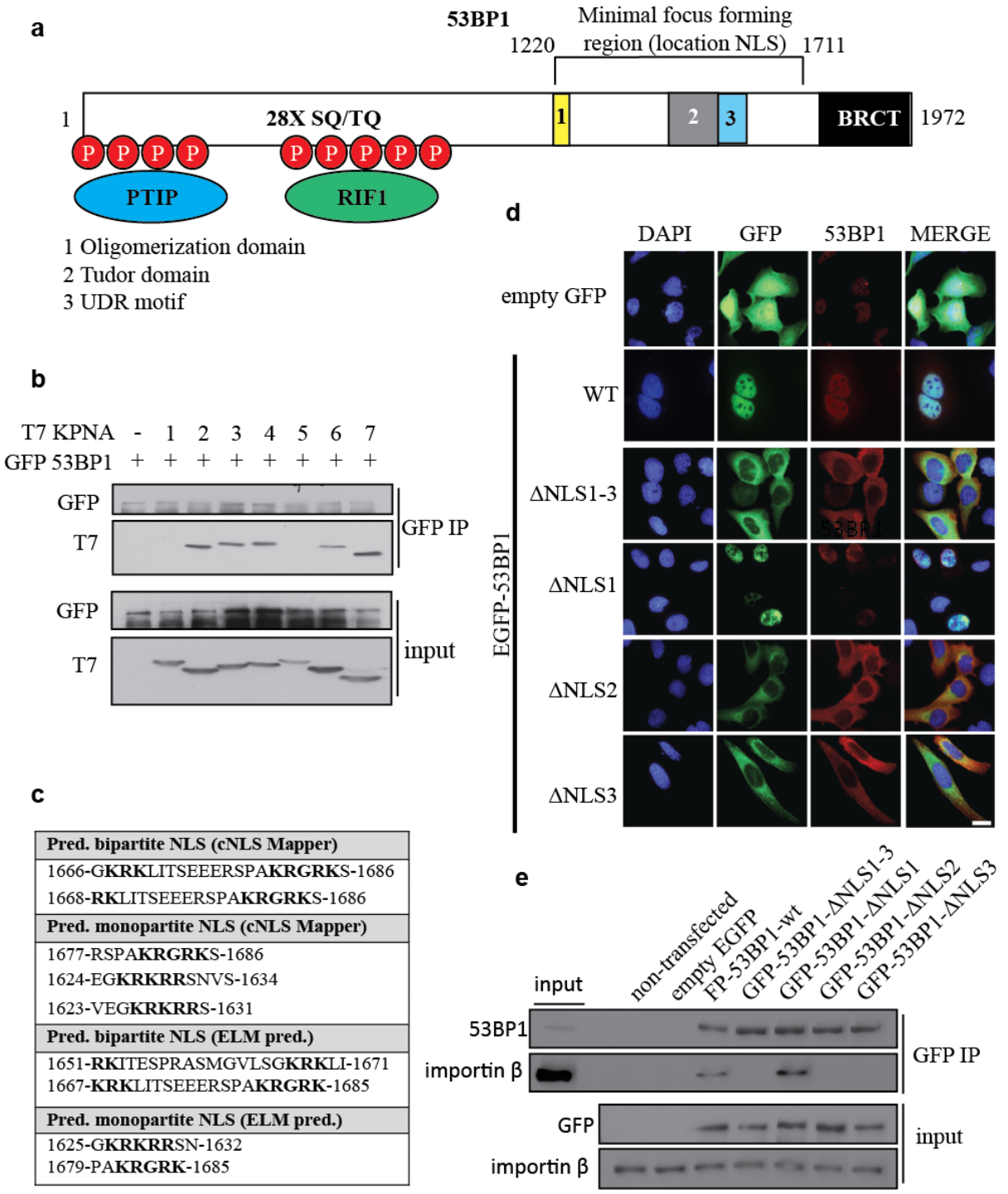

Figure 1. 53BP1 has a classical bipartite NLS.
(A) Graphic representation of 53BP1s functional domains. (B) CO-IP of GFP 53BP1 from HEK293 cells co-transfected with T7 tagged importin α. (C) Predicted (pred.) NLS sequences in 53BP1 by cNLS mapper and ELM prediction. Basic motifs are depicted in bold. (D) Microscopic analysis of localisation of GFP 53BP1 WT and GFP 53BP1 NLS mutants in U2OS cells. Scale bar 20 µm. (E) CO-IP of GFP 53BP1 WT and GFP 53BP1 NLS mutants from transiently transfected U2OS cells

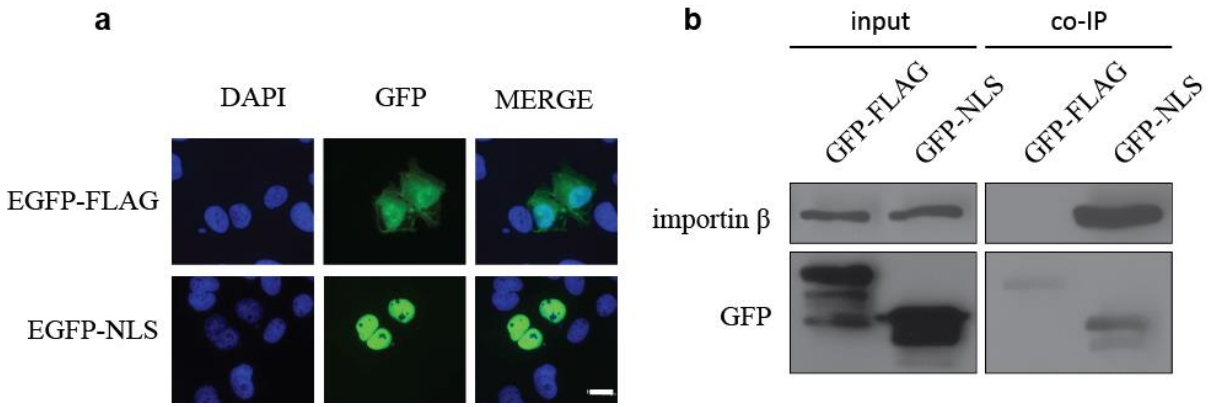

Figure 2. Bipartite NLS 1658-RASMGVLSGKRKLITSEEERSPAKRGRKSA-1687 is sufficient for nuclear localisation.

(A) Microscopic analysis of localisation of GFP FLAG construct and GFP with the bipartite 53BP1 NLS1658-RASMGVLSG**KRK**LITSEEERSPA**KRGRK**SA-1687. Scale bar 20 µm.
(B) CO-IP of GFP FLAG or GFP NLS construct from transiently transfected U2OS cells.

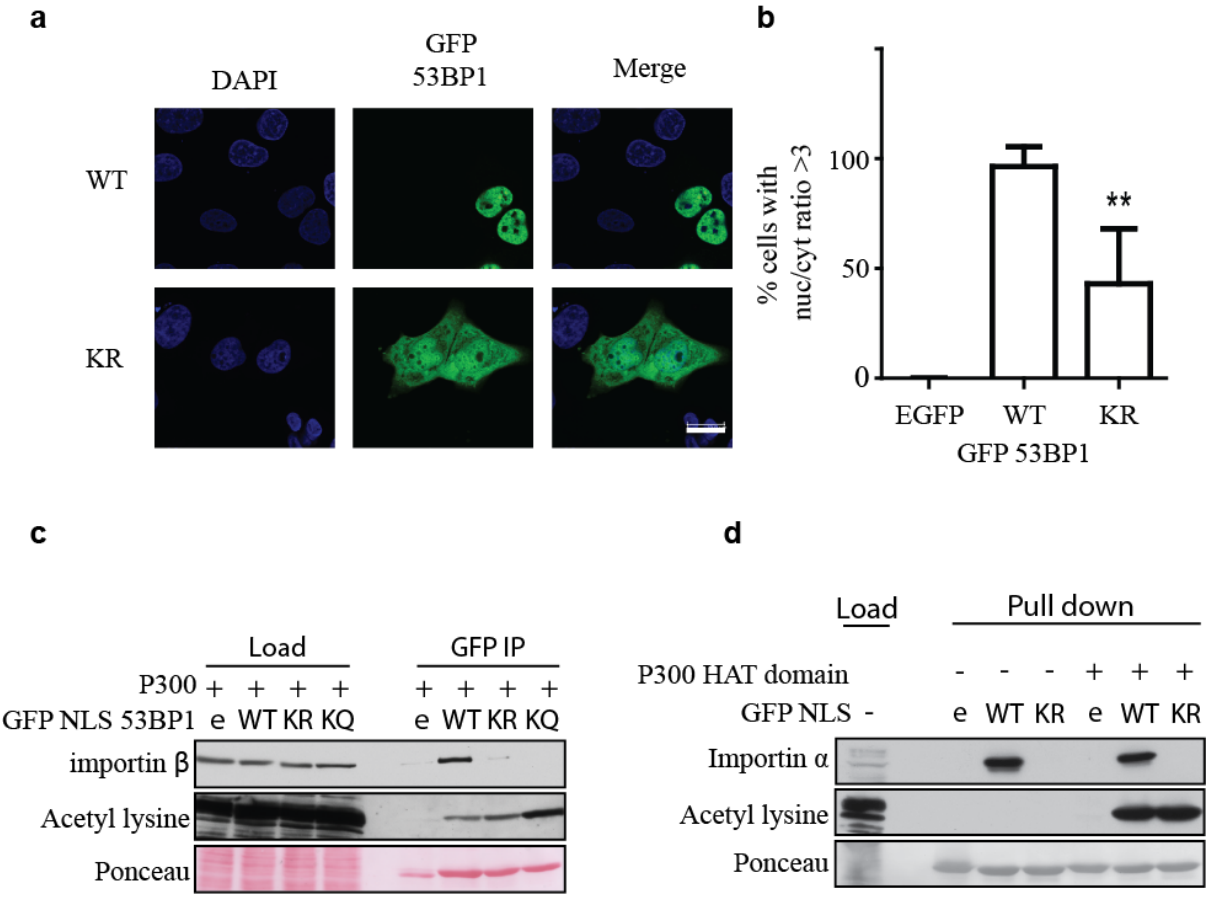

Figure 3. K1667 integrity is needed for importin binding and nuclear localisation of 53BP1.

(A) Microscopic analysis of localisation of GFP 53BP1 WT and GFP 53BP1 K1667R in transiently transfected U2OS cells. Scale bar 20 µm. (B) Quantification of A using 3 biological replicates and more than 15 cells per replicate. The graph shows the percentage of cells with a ratio of nuclear and cytoplasmic signal >3 ** = P value <0.01. (D) CO-IP of GFP NLS from HEK293 cells co-transfected with P300. (E) In vitro acetylation of GFP NLS and subsequent pull down from HEK293 whole cell lysate.

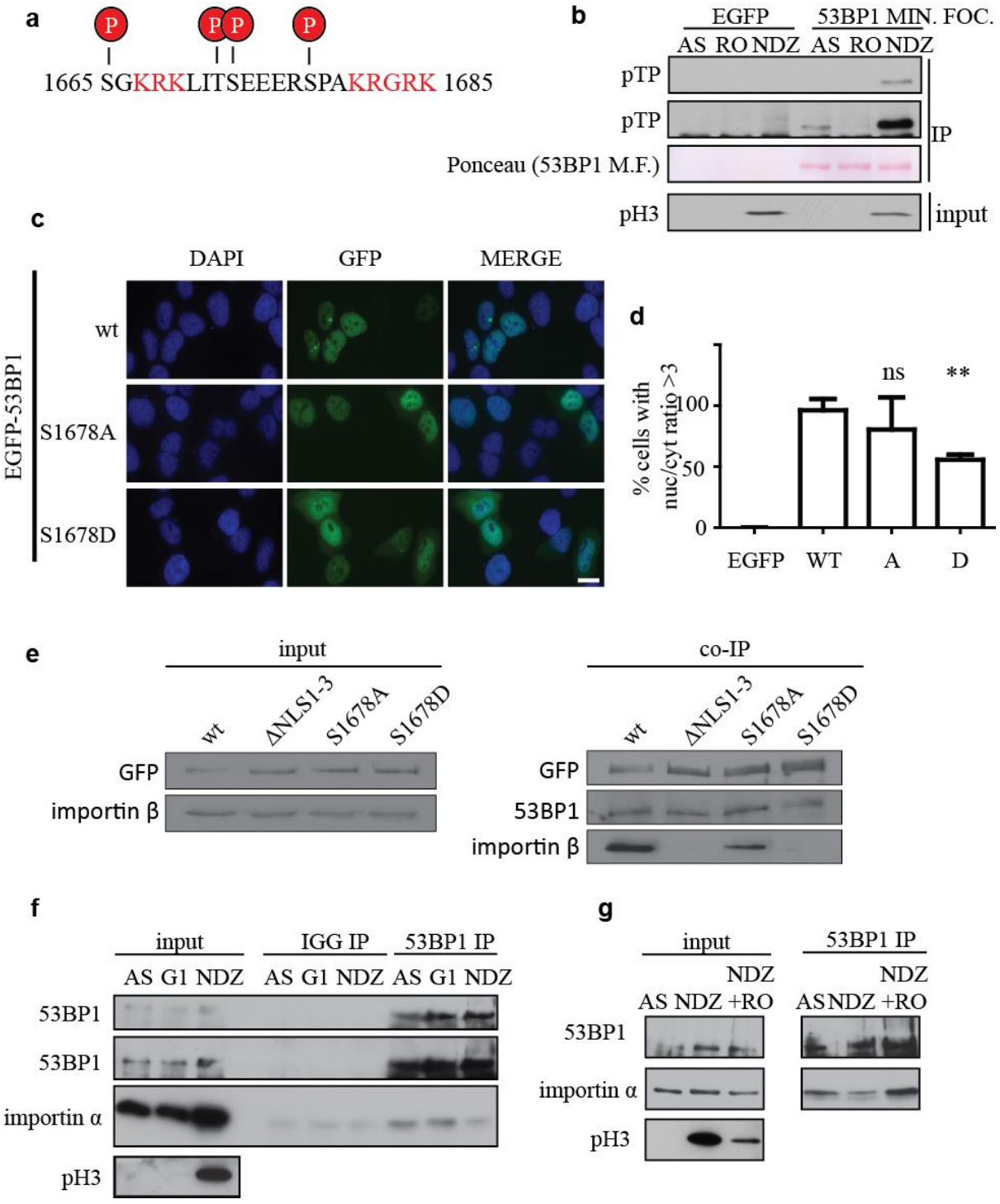

Figure 4. Regulation of 53BP1 nuclear import by a CDK1 phosphorylation site.
(A) 53BP1 bipartite NLS with phosphorylations as found in mass spectrometry screens. Scale bar 20 µm. (B) Microscopic analysis of localisation of GFP 53BP1 WT/S1678A/S1678D in U2OS cells. (C) Quantification of B using 3 biological replicates and more than 15 cells per replicate. The graph shows the percentage of cells with a ratio of nuclear and cytoplasmic signal >3 ns= not significant **= P value <0.01 (D) CO-IP of GFP 53BP1 WT/ΔNLS/S1678A/S1678D from U2OS cells.

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